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First survey for *Babesia bovis* and *Babesia bigemina* infection in cattle from Central and Southern regions of Portugal using serological and DNA detection methods

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ABSTRACT

Incidence of bovine babesiosis in Portugal is currently unknown. In this study, a first survey of *Babesia bovis* and *Babesia bigemina* infection in cattle was carried out using blood samples from 406 clinically healthy individuals from different districts from Central and Southern regions of Portugal and analyzed by indirect enzyme linked immunosorbent assay (iELISA) and nested polymerase chain reaction (nPCR). Overall, serological testing revealed that 79% and 52% of cattle were positive for *B. bovis* and *B. bigemina* antibodies, respectively, whereas nPCR testing detected 71% and 34% cattle infected with *B. bovis* and *B. bigemina* protozoan, respectively. This is the first report of the prevalence of *B. bovis* and *B. bigemina* in cattle obtained by serological and DNA analysis studies in Central and Southern regions of Portugal. These data suggests high incidence of *Babesia* sp. infection in Portugal and can be used for designing adequate control programs.

1. Introduction

Bovine babesiosis is a tick-borne disease caused by infection with intraerythrocytic Apicomplexa protozoan parasites from the genus *Babesia* that are distributed worldwide (McCrosker, 1981). *Babesia bovis* and *Babesia bigemina* are the most common species infecting cattle (Figueroa et al., 1998).

In Portugal there are at least three classes of ticks identified that are competent for transmission of *B. bovis* and *B. bigemina: Ixodes ricinus, Rhipicephalus bursa* and *Rhipicephalus* (*Boophilus*) *annulatus* (Caeiro, 1999; Estrada-Pena et al., 2004). The species *R. bursa* and *R. annulatus* are mostly restricted to the Southern part of the country under hot and dry climate conditions of mediterranean type. *I. ricinus* is present in Southern zones of the country, and also in Western parts where the climate is habitually humid (Estrada-Pena and Santos-Silva, 2005). Previous studies suggest the occurrence of *B. bovis* infections in Portugal (Caeiro, 1999; Criado-Fornelio et al., 2003), however the incidence and distribution of *Babesia* infections among the estimated 1.1 millions cattle population currently in Portugal remains unknown and systematic studies for determining the magnitude of these infections are needed for the development of a control strategy for babesiosis in this country.

Such studies required the use of state-of-the-art diagnostic tools that are both sensitive and specific. Accurate serological detection of *Babesia* infections was achieved using ELISA tests based on the C-terminus portion of the *B. bovis* and *B. bigemina* rhoptry-associated protein-1 (RAP-1) proteins (Boonchit et al., 2004, 2006; Goff et al., 2006, 2008). In addition, although these RAP-1 protein-based serological tests provided a good level for detection of early and late infections in cattle (Goff et al., 2003), and due to the time required for the emergence of detectable

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humoral immune responses, there is also the possibility of very early infections being undetected using only serological methods. In contrast, parasites in blood are usually more easily detectable during this early acute stage of the disease; therefore these cases can also be identified using highly sensitive nested PCR techniques (Goff et al., 1988; Brown et al., 1996; Calder et al., 1996; Dorigo-Zetsma et al., 1999; Birkenheuer et al., 2003; Miyama et al., 2005; Oyamada et al., 2005; Boonchit et al., 2006; Heim et al., 2007). This study was planned in order to survey the status of *B. bovis* and *B. bigemina* infections in selected Central and Southern regions of Portugal that were presumed endemic for *Babesia* infection. The survey was conducted by serological assessment for *B. bovis* and *B. bigemina* infections with an indirect ELISA (iELISA) based on RAP-1 antigens derived from *B. bovis* and *B. bigemina* Portuguese strains, and nested-PCR (nPCR) reactions on total DNA extracted from cattle blood samples.

2. Material and methods

2.1. Sampling of cattle blood

A total of 406 cattle blood samples were randomly collected under sterile conditions and with or without EDTA as anticoagulant between March 2006 and January 2007. The blood samples were collected from apparently clinically healthy cattle located in four different districts from Central and Southern regions of Portugal: 29 animals from 2 farms in Santarém (Central region), and 377 samples from 45 farms in Southern region [163 animals from 24 farms from Setúbal (Southern West of Portugal), 194 animals from 20 farms in Beja and 20 animals from 1 farm in Évora (Southern East of Portugal)] (map with the localization of the four districts is shown in Supp. data, Fig. 1). Blood samples from approximately 10% of the total number of cattle existent in the each farm were used in this study.

2.2. Processing of DNA and cattle blood sera

DNA was extracted from whole blood collected with EDTA as an anticoagulant using the Puregene DNA Purification System Blood Kit™ (Gentra/Qiagen), according to manufacturer's instructions, and stored at −20 °C until used.

Serum was obtained from the blood samples collected without anticoagulants. After centrifugation, the supernatant was incubated for 1 h at 37 °C with 50 μg/ml of *Escherichia coli* lysate in order to minimize non-specific reactivity of the sera with *E. coli* cells present in the coated recombinant antigen (Silva et al., 2008).

2.3. Production of recombinant (r)RAP-1/CT-STR protein from Portuguese B. bovis and B. bigemina strains

The *rap-1 carboxyl-terminal* of *B. bovis* (*B. bovis*-rap-1/CT-STR) and *rap-1 carboxyl-terminal* of *B. bigemina* (*B. bigemina*-rap-1/CT-STR) genes were amplified from genomic DNA from the *B. bovis* and *B. bigemina* isolates of infected Portuguese cattle by PCR, cloned into the pBAD/TOPO ThioFusion vector (Invitrogen), and transformed into TOP-10 *E. coli* cells. Purified recombinant proteins were used to develop the ELISA method for the detection of antibodies against *B. bovis* and *B. bigemina* in cattle sera and for immunoblots as described below. The cloning, expression and purification procedure was performed as described in detail by Silva et al. (2008).

2.4. Immunoblot

Monoclonal anti-*B. bovis*-rRAP-1, BABB75A4 (Palmer et al., 1991), and anti-*B. bigemina*-rRAP-1, 64/04.10.3 (Vidotto et al., 1995), antibodies were used in immunoblots against *B. bovis*-recombinant (r)RAP-1/CT-STR protein and *B. bigemina*-rRAP-1/CT-STR protein, respectively. In each case, 1 μg of recombinant protein was electrophoresed in a 4–20% Tris–HCl gradient SDS-PAGE gel (Bio-Rad) and transferred to 0.45 μm nitrocellulose membrane for 1 h at 100 V. Membranes were blocked with 200 μl of ELISA blocking buffer – 5% skim milk in PBST (phosphate buffer saline – 0.150 M NaCl, 0.027 M KCl, 0.570 M Na_2HPO_4, 0.003 M KH_2PO_4, pH 7.2 – with 0.1% of Tween 20) – for 1 h at room temperature in an orbital shaker. Membranes were then incubated for 40 min with monoclonal antibodies diluted 1:3200 in ELISA blocking buffer, washed three times for 10 min each with PBST, incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse bovine immunoglobulin (Ig) G (KPL) diluted 1:10,000 with PBST, for 30 min. After three washings for 10 min each with PBST, the blots were developed using a chemiluminescence ECL Plus kit (Amersham) and an X-ray film.

2.5. iELISA assays

Indirect ELISA was carried out as previously described by Silva et al. (2008) for the detection of anti-*B. bovis*-rRAP-1/CT-STR and anti-*B. bigemina*-rRAP-1/CT-STR antibodies in bovine sera samples.

Briefly, 96-well microtiter plates (Immuno Plate MaxiSorp™ Nunc International) were coated overnight at 4 °C with 100 μl of the *B. bovis*-rRAP-1/CT-STR protein at a concentration of 2 ng/μl per well in a coating buffer (15 mM Na_2CO_3; 35 mM Na_2HCO_3, pH 9.6). The plates were then incubated with 200 μl of ELISA blocking buffer for 2 h at 37 °C. Serum samples were diluted to 1:40 and 1:80 in PBST, added to a well, separately, and then incubated for 1 h at room temperature. After three washes with PBST, wells were incubated with 100 μl of HRP-conjugated mouse anti-bovine IgG (Sigma, diluted 1:1000 in PBST) for 1 h at room temperature. The plates were washed six times with PBST, and then 100 μl of the substrate solution, TMB (Pierce) was added to each well. After 30 min of incubation at room temperature, the reaction was stopped by the addition of 75 μl of stop buffer (2 M H_2SO_4). The mean optical density (OD) was measured at wavelength 450 nm using a Spectra max 340 (Molecular Devices). The OD_{450} cut-off was set as the mean value of the 12 negative bovine sera plus three standard deviations.

An identical iELISA protocol was performed using *B. bigemina*-rRAP-1/CT-STR purified recombinant protein.

2.6. Nested PCR assays

PCR amplification was carried out on genomic DNA extracted from cattle blood samples, and analyzed by nPCR for the screening of *B. bovis* and *B. bigemina*, using a previously described protocol (Figueroa et al., 1992, 1993).

Briefly, PCR assay was performed in a total volume of 25 μl using GoTaq Green Master Mix (Promega Corporation) following the manufacturer's protocol, using 1 μM of each primer and 5 μl of genomic DNA. The same GoTaq Green Master Mix and 5 μl of the external PCR amplified products were used for nested PCR (primers sets are listed in Supp. data, Table 1). Genomic DNA from *B. bovis* Mo7 strain and genomic DNA from *B. bigemina* S1A strain were used as positive controls for *B. bovis* and *B. bigemina* PCR reaction, respectively. An additional contamination control was used for each set of reactions, where genomic DNA was replaced by an equal volume of sterile distilled water.

The amplified PCR products were subjected to electrophoresis on 2% agarose gels, stained with ethidium bromide, visualized under UV light, and photographed in ChemiDoc XRS (BIO-RAD) apparatus. The amplicons' molecular sizes were estimated by including a base pair standard (GeneRuler™ 100 bp DNA Ladder-Fermentas). Samples presenting visible bands of approximately 290 bp and 170 bp were considered positive for *B. bovis* and for *B. bigemina*, respectively.

2.7. Sequencing

To further confirm and validate the PCR results, randomly selected positive samples from *B. bovis* and positive samples from *B. bigemina* were sequenced. For this purpose, 50 μl of the PCR products were purified from the agarose gel with the SV Gel and PCR Clean-Up System Kit (Promega) following the instructions of the manufacturer. The purified amplicons from *B. bovis* were sequenced with the *BoFN–BoRN* primers (Figueroa et al., 1993) and the purified amplicons from *B. bigemina* with the *BilAN–BilBN* primers (Figueroa et al., 1992).

2.8. Statistical analysis

Chi-square test was used to evaluate the hypothesis for significant difference between the infection in different locations and cattle breed. P values less than 0.05 ($P < 0.05$) were considered statistical significant. Chi-square (χ^2) values and degrees of freedom (df) are also presented.

3. Results

3.1. Characterization of rRAP-1/CT antigens derived from Portuguese B. bovis and B. bigemina Santarém (STR) isolates and validation of iELISA serodiagnostic tests

The *B. bovis-rap-1/CT-STR* and *B. bigemina-rap-1/CT-STR* genes were amplified from genomic DNA isolated from *Babesia*-infected Portuguese cattle from Santarém region. The amplicons were cloned into pBAD/TOPO ThioFusion vector (Invitrogen), sequenced to confirm the correct gene orientation, expressed and purified. Sequence comparisons showed 98% identity between the *B. bigemina-rap-1/CT-STR* nucleotide sequence and the published *B. bigemina rap-1* (*p58*) gene (GenBank accession no.: M60878) (Goff et al., 2008). The sequence analyzes indicate that *B. bovis* and *B. bigemina-rap-1/CT-STR* genes are highly conserved among American and European strains tested (data not shown). The characterization of the *B. bovis*-rRAP-1/CT-STR protein and validation of a *B. bovis* indirect ELISA (iELISA) test was described previously (Silva et al., 2008).

To determine the conservation of previously defined B-cells epitopes in the *B. bovis* and *B. bigemina*-rRAP-1/CT-STR proteins, it was tested by immunoblots the reactivity of these two proteins with the mouse monoclonal antibodies BABB75A4 (Palmer et al., 1991) and 64/04.10.3 (Vidotto et al., 1995) which are reactive with *B. bovis* and *B. bigemina*-rRAP-1 proteins respectively. Monoclonal anti-*B. bovis*-rRAP-1 antibody, reacted with only one antigen of 75 kDa *B. bovis*-rRAP-1/CT-STR protein (Fig. 1A, lane 2), and the monoclonal anti-*B. bigemina*-rRAP-1 antibody also reacted with only one antigen of 36 kDa in *B. bigemina*-rRAP-1/CT protein (Fig. 1B, lane 4). In both cases, the sizes of the antigens recognized by the monoclonal antibodies is consistent with the expected molecular weighs of the rRAP-1 fragments of *B. bovis* and *B. bigemina* respectively, and no antigen recognition whatsoever was observed when the two recombinant proteins were incubated with pre-immune mouse sera as a negative

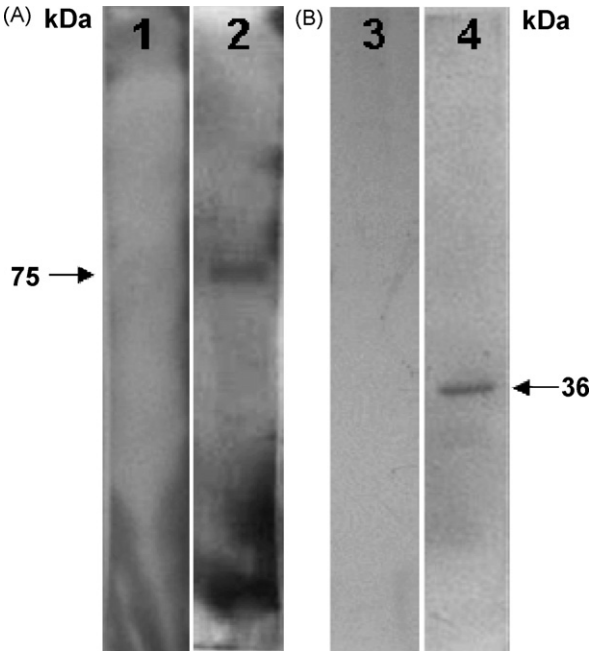

Fig. 1. Recognition of *B. bovis*-rRAP-1/CT-STR and *B. bigemina*-rRAP-1/CT-STR proteins by RAP-1 protein monoclonal antibodies. (A) *B. bovis*-rRAP-1/CT-STR protein incubated with: pre-immune mouse sera (lane 1) and monoclonal anti-*B. bovis* RAP-1 protein antibodies, BABB75A4 (lane 2). (B) *B. bigemina*-rRAP-1/CT-STR protein incubated with: pre-immune mouse sera (lane 3) and monoclonal anti-*B. bigemina* RAP-1 protein antibodies, 64/04.10.3 (lane 4). Blots were incubated with HRP-conjugated goat anti-mouse IgG and developed by ECL detection. Individual bands and their corresponding molecular masses (kDa) are indicated by arrows.

control (Fig. 1A, lane 1 and Fig. 1B, lane 3). Thus, it is possible to conclude that these two previously defined monoclonal antibody defined B-cells epitopes that are used for the detection of *Babesia* infection in cattle using standardized and validated ELISA methods (Boonchit et al., 2004, 2006; Goff et al., 2006, 2008) are also conserved in the *Babesia* sp. isolated in Santarém. In addition, binding of these two monoclonal antibodies to the recombinant versions of the *B. bovis* and *B. bigemina* RAP-1 proteins was demonstrated in an ELISA. Importantly, no antibody cross-reactivity between *B. bovis* and *B. bigemina* was observed, therefore the data confirmed that these two RAP-1 derived recombinant proteins can be used as specific serological reagents for surveying the occurrence of *B. bovis* and *B. bigemina* in Portugal, as described below.

3.2. Detection of *B. bovis* and *B. bigemina* infection in Portuguese cattle

A total of 406 cattle samples randomly collected from Central and Southern regions of Portugal were analyzed by both iELISA and nPCR techniques. The data, presented in Table 1, show that the *B. bovis*-rRAP-1/CT-STR iELISA detected a total of 322 positive bovine samples (79.3%) whereas the *B. bigemina*-rRAP-1/CT-STR iELISA detected 211 positive bovine samples (52%). A similar pattern was observed when these samples were analyzed by nPCR, with 287 bovine samples positive for *B. bovis* (70.7%), and 139 positive for *B. bigemina* (34.2%) (Table 1). Mixed infections of *B. bovis* and *B. bigemina* were detected in a total of 181 animals (44.6%) by iELISA and in a total of 103 animals (25.4%) by nPCR (Table 1). *B. bovis* single infections were detected in a total of 141 animals (34.7%) by iELISA and in a total of 184 animals (45.3%) by nPCR. *B. bigemina* single infections were detected in a total of 30 animals (7.4%) by iELISA and in a total of 36 animals (8.9%) by nPCR. Overall, these data indicate a significantly higher presence of infection with *B. bovis* than with *B. bigemina*, with a large number of co-infected cattle, in these Portuguese regions, in a trend that could be consistently detected using two distinct diagnostic tests.

In the course of this study, the distribution of *B. bovis* and *B. bigemina* infections among cattle breeds was also compared. Not statistically significant differences ($P < 0.05$) were found among the 5 cattle breeds involved (Charolais crossbred, Limousin crossbred, Limousin, Mertolenga and Friesian) (data not shown).

Sequence comparisons among the *rap-1 B. bovis* genes amplified revealed between 98 and 100% of sequence identity among all Portuguese isolates for *B. bovis* (GenBank accession nr: FJ901342; FJ939723). Thus, consistent with previous observations, the sequences of *B. bovis rap-1* gene from Portuguese isolates are highly conserved and similar to the published *B. bovis* sequence from the Argentina strain (R1A, GenBank accession nr: AF030055), Texas strain (T2Bo, GenBank accession nr: AF030059) and Australian strain (S2P, GenBank accession nr: AF030056) (Suarez et al., 1993, 1994; Brown et al., 1996; Suarez et al., 1998). In contrast, although the sequenced amplicons from *B. bigemina* showed 100% sequence identity among the Portuguese isolates for *B. bigemina* (GenBank accession nr: FJ939724), the amplicons obtained from the *B. bigemina* Portuguese isolates had 82% identity with the published *SpeI–AvaI B. bigemina* restriction fragment (GenBank accession nr: S45366) (Figueroa et al., 1992).

3.3. Geographic distribution of *B. bovis* and *B. bigemina* infection in cattle from Central and South regions of Portugal

B. bovis and *B. bigemina* infected cattle were found in all Portuguese regions analyzed although data suggest differences in the distribution of *B. bovis* and *B. bigemina* infections among some regions.

The serologic and molecular results describing the detection of *B. bigemina* infected cattle among distinct regions are represented in Fig. 2A. Serological *B. bigemina* iELISA analysis (Fig. 2A) pointed out Évora as the region with the lowest number of infected animals (35%) and Setúbal as the region with the highest percentage of infected animals (55.2%) ($\chi^2 = 13.0$, $df = 3$, $P < 0.05$). Nested PCR analysis detected mostly *B. bigemina* parasites in Beja region, with 37.1% of animals infected. Consistent with the serological data, nPCR analysis detected the lowest *B. bigemina* rate of infection in Évora region (10%) ($\chi^2 = 19.7$, $df = 3$, $P < 0.05$).

The serologic and molecular results obtained for *B. bovis* infections in cattle from several areas of Portugal are represented in Fig. 2B. The lowest percentage of cattle with detectable antibodies against *B. bovis* was found in the

Table 1
Distribution and frequency (%) of *Babesia* sp. in 406 samples of cattle DNA and serum from analyzed by iELISA and nPCR technique.

	Babesia sp.	iELISA		nPCR	
		Number	Percent	Number	Percent
Single infection	*B. bovis*	141	34.7	184	45.3
	B. bigemina	30	7.4	36	8.1
Mixed infection	*B. bovis* + *B. bigemina*	181	44.6	103	25.4
Total infection	*B. bovis*	322	79.3	287	70.7
	B. bigemina	211	52.0	139	34.2
Negative		54	13.3	83	20.4
Total analyzed		406		406	

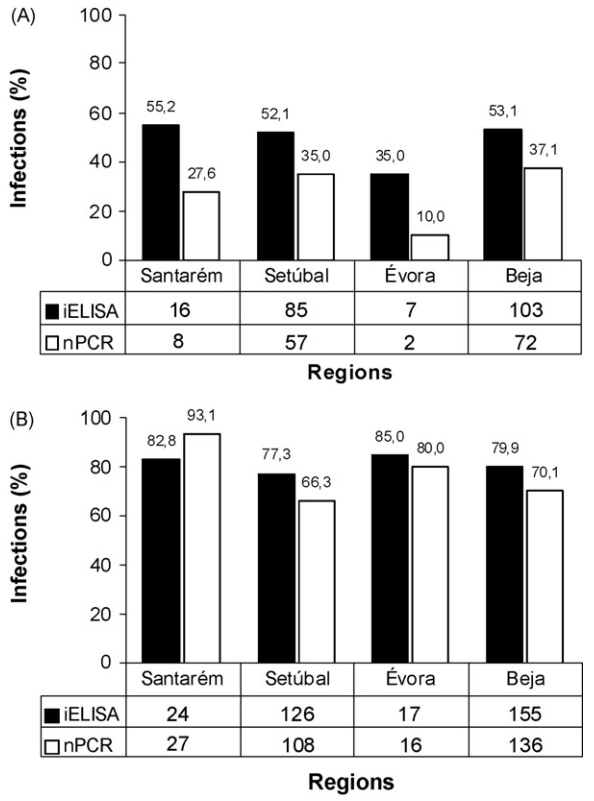

Fig. 2. Analysis of *Babesia* sp. infection by iELISA and nPCR techniques among cattle samples from the four district-based studied: Santarém ($n = 29$), Setúbal ($n = 163$), Évora ($n = 20$) and Beja ($n = 194$). (A) Distribution and frequency (%) of infected *B. bigemina* cattle. (B) Distribution and frequency (%) of infected *B. bovis* cattle.

Setúbal region (77.3%), whereas the highest percentage was detected in the Évora region (85%) (Fig. 2B). However, the serological differences found for the geographical distribution of *B. bovis* infection in Portugal were not significant ($\chi^2 = 1.05$, $df = 3$, $P > 0.05$). Nested PCR analysis revealed that *B. bovis* parasites are present at the highest percentage in cattle from the Santarém region (93.1%) and with the lowest percentage of infection in Setúbal region (66.3%), with evidence of statistical difference ($\chi^2 = 9.45$, $df = 3$, $P < 0.05$) (Fig. 2B).

4. Discussion

In this study, serological and molecular tools were used for the first time to detect *B. bovis* and *B. bigemina* in cattle blood from the previously uncharacterized Central and Southern regions of Portugal. Recombinant carboxyl-terminal-RAP-1 proteins (*B. bovis*-rRAP-1/CT-STR protein and *B. bigemina*-rRAP-1/CT-STR protein) were used to detect antibodies against *B. bovis* and *B. bigemina* in cattle sera samples by iELISA, and nPCR was used to amplify the conserved *rap-1 B. bovis* gene and *SpeI–AvaI B. bigemina* fragment in genomic DNA (Figueroa et al., 1992, 1993, 1994). These two methods have been previously reported to have high specificity and sensitivity for their use in epidemiology surveys (Figueroa et al., 1993; Goff et al., 2006, 2008). The results of this study indicate a high presence of infection of cattle by *B. bovis* and *B. bigemina* parasites in the surveyed areas and suggest that proper preventive measures will be required for the control of this costly disease.

Serologic assays detected a higher number of infected *Babesia* sp. cattle than nPCR. These differences are consistent with previous findings that *B. bovis* and *B. bigemina* parasites are difficult to detect using PCR techniques due to the small number of parasites that occur in peripheral blood during chronic infections (Pipano et al., 2002); another possible reason for the high number of cattle with detectable *Babesia* sp. antibodies could be that these antibodies remain in circulation for a long period of time after acute infection. In such cases, it is possible, either that the parasites are cleared from circulation, or that the parasite concentration in the blood drops below the limit of detection of the nPCR (Tjornehoj et al., 1996; Pipano et al., 2002). Before this research was conducted, no reliable diagnostic studies were pursued by the incumbent farmers despite bovines showing signs consistent with *Babesia* clinical disease. Therefore, there were no previous reports on *B. bovis* and *B. bigemina* clinical manifestations in the regions of Portugal analyzed in this study, and the history of occurrence of clinical cases in these farms remains unknown.

Nucleotide sequence comparisons of several *rap-1* from Portuguese *B. bovis* field isolates showed a higher level of sequence conservation when compared to the Argentina, Texas and Australia *B. bovis* strains. The *SpeI–AvaI B. bigemina* nucleotide sequences obtained from the Portuguese isolates revealed some level of polymorphism when compared to the previously published strain. In contrast, the nucleotide sequence of this fragment showed high conservation within *B. bigemina* isolates from North and Central America and the Caribbean Region (Figueroa et al., 1992). No previous information was available on the conservation of the *SpeI–AvaI* fragment in *B. bigemina* isolates from Europe, suggesting that more European isolates need to be collected and sequenced to further test the level of nucleotide conservation of this *SpeI–AvaI B. bigemina* DNA fragment.

The antibody prevalence has been used as an indicator of endemic stability or instability situations (Mahoney and Goodger, 1972; Carrique et al., 2000). Overall, anti-*B. bovis* antibodies were detected in over 75% of the cattle analyzed, suggesting that all the regions analyzed in this study might be in an endemically stable situation. Thus, it is possible that cattle may have developed immunity to the parasite, decreasing the occurrence of detectable clinical babesiosis cases (Homer et al., 2000). Conversely, it is also possible that an endemically unstable situation occurs for *B. bigemina* where less than 75% of the cattle are seropositive, in a scenario in which cattle may be at a significantly higher risk of clinical disease (L'Hostis and Seegers, 2002).

5. Conclusions

The present study provides the first survey of *B. bovis* and *B. bigemina* in cattle from Central and Southern regions

of Portugal. Evidence of *B. bovis* and *B. bigemina* infection could have an important impact in weight loss, milk production and possible abortion (Wagner et al., 2002).

The outcomes of this preliminary study suggests the need for the implementation of a more complete study assessing the epidemiological situation of bovine babesiosis in Portugal, including testing animals from all Portuguese regions and an evaluation of the relationships between the disease and animal ages, breeds, environmental conditions, and tick vector prevalence. These data will be of importance in order to carry out a comprehensive and rationally designed plan to control and prevent these currently neglected diseases, at a regional level.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2009.07.031.

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